Paper chromatography of sugar phosphates and three-carbon phosphates. Extension and modification of the Agarwal procedure*

Many methods[1–6] have been developed for the paper chromatographic separation of phosphorylated metabolic intermediates. While using one of these procedures[6], originally designed for the chromatography of hexose phosphates, in the study of organic phosphates in honey[7], it was found that this procedure would also separate sugar phosphates from some three-carbon phosphates as a group. In addition, with the modification described here, some separation of the individual three-carbon phosphates was obtained.

Experimental

Reagents. All reagents were analytical grade and used as supplied.

Standard sugar phosphates and three-carbon phosphates. These standards were converted to their ammonium salts by the method of AGARWAL *et al.*[8]. The amount of salt used produced a 0.05 *M* solution of free acid or ester. The standards are listed with their name, source and abbreviation used in the text**.

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** Mention of trade or company names does not imply endorsement by the Department over others not named.

Fructose 1-phosphate, barium salt, Sigma Chemical Co.	F-1-P
Fructose 6-phosphate, barium salt, Sigma Chemical Co.	F-6-P
Fructose 1,6-diphosphate, barium salt, courtesy of J. W. WHITE, Jr.	F-1,6-P
Glucose 1-phosphate, dipotassium salt, Calbiochem	G-1-P
Glucose 6-phosphate, disodium salt, Calbiochem	G-6-P
Ribose 5-phosphate, sodium salt, Sigma Chemical Co.	R-5-P
Dihydroxyacetone phosphate, dimonocyclohexylamine salt, Sigma Chemical Co.	DHAP
2,3-Diphosphoglyceric acid, barium salt, Sigma Chemical Co.	2,3-PGA
DL-Glyceraldehyde 3-phosphate, diethyl acetal, barium salt, Sigma Chemical Co.	G-3-P
DL-α-Glycerophosphate, disodium salt, hexahydrate, Sigma Chemical Co.	α-GP
DL-β-Glycerophosphate, disodium salt, hexahydrate, Sigma Chemical Co.	β-GP
2-Phosphoglyceric acid, barium salt, Sigma Chemical Co.	2-PGA
3-Phosphoglyceric acid, barium salt, Nutritional Biochem. Corp.	3-PGA
Phospho(enol)pyruvic acid, barium salt, monohydrate, Sigma Chemical Co.	PEP

Solvent systems. AGARWAL formic acid[6]: *tert.*-butanol–50 % formic acid–water (16:1:4).

ALBON AND GROSS[9]: *n*-propanol–ethyl acetate–water (7:1:2).

Spray reagent. The molybdate reagent of HANES AND ISHERWOOD[1] was used to locate the phosphate spots.

Apparatus. Glass tanks, 61 cm high × 30 cm square, designed for descending chromatography and accommodating 4 glass troughs, each holding a 21 × 56 cm chromatogram.

Paper washer, 49.5 × 61 × 16.5 cm (made of Plexiglas) with a perforated plate 8.5 cm from the bottom.

Whatman No. 1 filter paper, after proper washing, was used for all chromatography.

General Electric 15-W germicidal ultraviolet lamp. Papergrams were irradiated with this lamp[2] to produce the blue phosphomolybdate spots.

Purification of the filter paper. The filter paper washing was a very critical step, because calcium and magnesium salts in the paper cause irreversible adsorption of the phosphates[10] and retard their migration. Two methods of washing were used: the first involved soaking in formic acid (1 *N*) followed by a soak in 0.5 % versene, pH 8.5 (ref. 5); the second omitted the soak in versene.

Chromatographic procedures

(1) *General.* All chromatograms were developed in the descending direction at 25–30°.

A uniform atmosphere was provided in the tank by use of solvent-wetted sheets (10 × 26.5 cm) of Whatman No. 3MM paper around the sides at the bottom. In addition, a blank sheet of Whatman No. 1 paper was suspended from one of the troughs and developed in the same manner as the chromatograms. The tank was al-

lowed to equilibrate at least 4 h for the fast-moving propanol system and overnight for the slower-moving formic acid system. A serrated edge was cut into the bottom end of the paper so that the solvent could flow off evenly.

The phosphates were used as their ammonium salts. Well defined spots were obtained when a 3 μl portion of a 0.05 *M* (as free acid or ester) solution was applied to the paper. Nine compounds, spotted 2 cm apart, could be studied at one time on a 21 × 56 cm chromatogram. The distance traveled was measured from the origin to the center of the spot. Orthophosphate (2 μl of 0.05 *M* $Na_3PO_4 \cdot 12H_2O$) was spotted on every chromatogram as a reference standard. The distance traveled by the organic phosphates was compared with the distance traveled by the orthophosphate. This ratio is the position constant, *Rp*.

(*2*) *Original Agarwal procedure.* The original AGARWAL procedure[6] was followed with two exceptions. Phosphates were applied as ammonium salts and formic acid-washed paper was used.

(*3*) *Modified Agarwal procedure.* The chromatogram was developed on formic acid-washed paper using successive irrigations with the propanol system and the formic acid system. First, the paper was put into the tank containing the propanol system and, after a 2-h equilibration, was irrigated for 15.5 h. The solvent was allowed to run off the paper. The sheet was removed and air-dried in a hood for 3 h. It was then put into the formic acid system tank, equilibrated 4 h, and irrigated for 20 h in the same direction traveled by the propanol system. This solvent front was not allowed to run off the paper. The sheet was removed when the front reached the beginning of the serrated edge.

Detection of phosphates. The phosphates were located with the molybdate reagent in the following way. Papers were heated at 85–90° for 3–5 min, then sprayed thoroughly, but not soaked, to insure complete hydrolysis of some of the very stable three-carbon phosphates. Papers were allowed to air-dry completely then heated again as above for about 1 min. The papers were carefully watched during this heating. If they began to change color, the heating was discontinued. After heating, the papers were illuminated with the U.V. lamp at a distance of about 15 cm until blue spots appeared against a white or buff background. Inorganic phosphate produced a blue-green spot and G-1-P showed a blue-green-purple spot. All of the other organic phosphates showed blue spots.

Results

In the original AGARWAL formic acid system[6], α-GP, 2-PGA and 3-PGA all moved the same distance. β-GP moved just a little faster (Fig. 1). When the development with AGARWAL formic acid system was preceded by irrigation with the ALBON AND GROSS propanol system[9], the glycerophosphates moved ahead of the phosphoglyceric acids. In addition, the β-GP traveled faster than the α-GP. The 2-PGA and 3-PGA showed a slight separation with 2-PGA moving a little faster (Fig. 2). These effects are also shown by the position constants listed in Table I.

In the original AGARWAL procedure, both G-1-P and G-6-P produced double spots whose movement was highly reproducible (Fig. 1). When the propanol treatment was used G-1-P still formed double spots consistently, but G-6-P now produced a single spot (Fig. 2). The movement of the hexose phosphates after the propanol irrigation was in the same order as that found by AGARWAL[6].

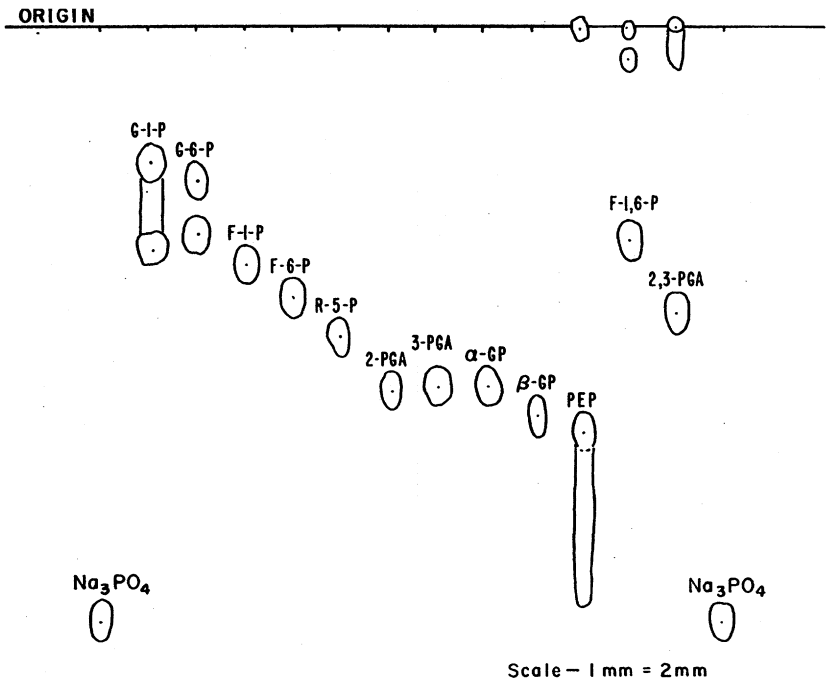

Fig. 1. Paper chromatogram of sugar phosphates and three-carbon phosphates developed in AGARWAL system for 39.5 h. Formic acid-washed paper was used.

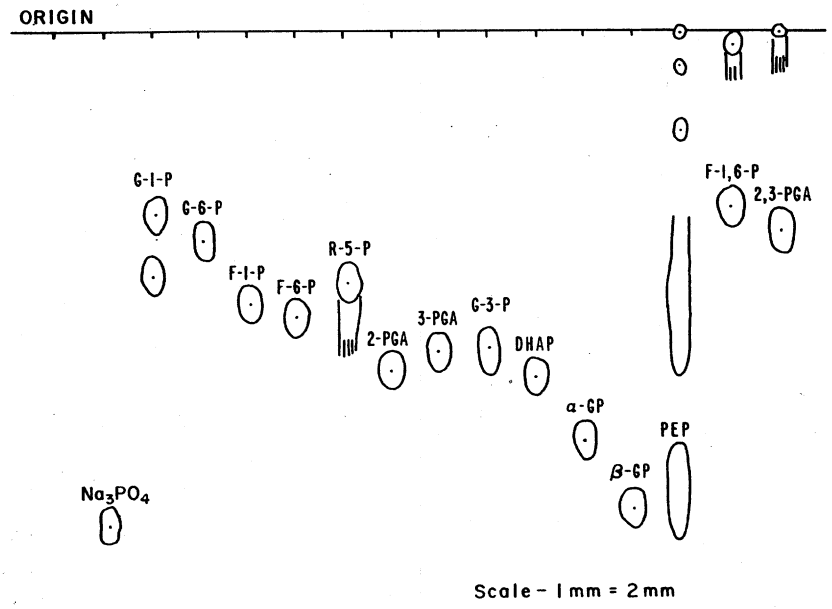

Fig. 2. Paper chromatogram of sugar phosphates and three-carbon phosphates irrigated with ALBON AND GROSS system for 15.5 h, then developed in AGARWAL system for 20 h. Formic acid-washed paper was used.

TABLE I

EFFECT OF SUCCESSIVE DEVELOPMENT WITH ALBON AND GROSS PROPANOL SYSTEM AND AGARWAL FORMIC ACID SYSTEM ON SEPARATION OF SUGAR PHOSPHATES FROM THREE-CARBON PHOSPHATES

Phosphate material	*Rp**	
	Agarwal solvent only	*Albon and Gross solvent Agarwal solvent*
G-1-P	23, 38 **	37, 53 **
G-6-P	26, 37 **	44
F-1-P	43	55
F-6-P	48	58
R-5-P	52	52
DHAP	—	74
G-3-P	—	67
2-PGA	57	72
3-PGA	57	69
α-GP	57	86
β-GP	64	99

* Figures are averages except for F-1-P, F-6-P, R-5-P, DHAP and G-3-P, which were from one run only; *Rp*, position constant $= \frac{\text{distance traveled by phosphate material}}{\text{distance traveled by orthophosphate}}$.

** Two spots from one origin.

The diphosphates, F-1,6-P and 2,3-PGA, formed multiple spots whose travel was unpredictable in both procedures.

The rate of travel of all of the phosphate compounds, including orthophosphate, was greatly increased by the irrigation with the propanol system. The propanol system alone did not move the phosphate compounds significantly from the origin. When the paper was irrigated with the propanol system before the phosphates were applied, there was no separation of three-carbon phosphates from each other. Their rate of travel was the same as that shown when the formic acid system alone was used.

Results were unsatisfactory when acid–versene-washed paper was used instead of formic acid-washed paper. All of the phosphates decomposed except 2-PGA, 3-PGA, β-GP and G-6-P.

The AGARWAL ammonia system[8] did not separate sugar phosphates from three-carbon phosphates and a preliminary irrigation with the propanol system had no effect.

Discussion

The propanol system irrigation probably made the conditions of chromatography less acidic than they were when the formic acid system was used alone. Hence there would be enough phosphate groups ionized to allow the solubility of the three-carbon phosphates to influence their migration. The sugar phosphates moved more slowly than the three-carbon phosphates, because the environment was still acid enough for the non-phosphate moieties to govern their rate of travel.

In addition to these chemical effects, the propanol system, which was designed originally for the chromatography of disaccharides[9], may have washed some carbohydrate impurities out of the paper or out of the phosphate samples themselves.

Several thin-layer methods for the separation of sugar phosphates were considered. The method of DIETRICH *et al.*[11], using ECTEOLA layers, was tried and it produced multiple spots for the three-carbon phosphates. The methods of WARING AND ZIPORIN[12] and SANDERSON *et al.*[13] were not applicable, because of reported insufficient separations and, therefore, were not investigated further. The proposed paper procedure provides a well-defined separation due to greater migration distance, and is simpler to use. About the same time is required as for preparation of plates and analysis with the thin-layer methods.

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1 C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
2 R. S. BANDURSKI AND B. AXELROD, *J. Biol. Chem.*, 193 (1951) 405.
3 D. C. MORTIMER, *Can. J. Chem.*, 30 (1952) 653.
4 H. E. WADE AND D. M. MORGAN, *Biochem. J.*, 60 (1955) 264.
5 V. C. RUNECKLES AND G. KROTKOV, *Arch. Biochem. Biophys.*, 70 (1957) 442.
6 D. P. AGARWAL, G. G. SANWAL AND P. S. KRISHNAN, *Analyst*, 87 (1962) 758.
7 M. H. SUBERS, A. I. SCHEPARTZ AND R. P. KOOB, *J. Apicult. Res.*, in press.
8 D. P. AGARWAL, G. G. SANWAL AND P. S. KRISHNAN, *Analyst.*, 88 (1963) 969.
9 N. ALBON AND D. GROSS, *Analyst.*, 77 (1952) 410.
10 A. A. BENSON, in S. P. COLOWICK AND N. O. KAPLAN (Editors), *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 111.
11 C. P. DIETRICH, S. M. C. DIETRICH AND H. G. PONTIS, *J. Chromatog.*, 15 (1964) 277.
12 P. P. WARING AND Z. Z. ZIPORIN, *J. Chromatog.*, 15 (1964) 168.
13 A. SANDERSON, C. E. BODWELL AND A. M. PEARSON, *Nature*, 206 (1965) 938.

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